IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Boyle, William J.

Serial No.: 09/211,315 Group Art Unit No.: 1644

Filed: December 14, 1998 Examiner: Turner, S.

For: Osteoprotegerin Binding Proteins

Docket No.: A-451G

DECLARATION OF JOHN K. SULLIVAN

Assistant Commissioner for Patents
Box AF
Washington, D.C. 20231

Sir:

I, John K. Sullivan, declare and state that:

1. I am presently employed by Amgen Inc. as an Associate Scientist in the Department of Inflammation and have held this position since May of 1991. My research interests are in the area of rheumatology and immunobiology.

2. I received my B.S. from Michigan Technological Institute in 1982 and a M.S. in Biology from Wright State University in 1984.

3. I have been asked to provide evidence concerning whether, based on the disclosure of U.S. Serial No. 09/211,315 (hereafter the '315 application), one of skill in the art without undue experimentation would have been able to obtain antibodies which bind osteoprotegerin binding protein (OPGbp) and modulate

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231, on the date appearing below.

August 18, 2000
Date

Sherry St. Andrew by Lynne Burkhaus
Signature

the activity of OPGbp such bone resorption is inhibited. The evidence presented in this declaration shows that the teachings of the '315 application would have enabled one to obtain antibodies which are suitable for use in the methods claimed in the '315 application. My conclusion is that the '315 application enables one skilled in the art to carry out the claimed methods without undue experimentation.

4. In arriving at this conclusion, I have read and understood the work of Dr. William J. Boyle in the '315 application, including Example 3 which describes the sequencing of DNA encoding murine OPGbp, Example 5 which describes the cloning and sequencing of DNA encoding human OPGbp, Examples 8 and 9 which describe *in vitro* and *in vivo* assays for OPGbp activity, and Example 11 which describes materials and procedures for the preparation of anti-OPGbp antibodies. Based on the work of Dr. Boyle in the '315 application, work done under my direction and control as set forth herein, and work done by others which is set forth herein based upon information provided to me, I conclude that the '315 application enables one skilled in the art to carry out the claimed invention without undue experimentation.

5. The following OPGbp peptides and polypeptides were used in the experiments described herein:

A BB' loop-Cys peptide having the amino acid sequence set forth on p. 47, line 18 of the specification and in SEQ ID NO:33 (hereafter "BB' loop-Cys").

A EF loop6-Cys peptide having the amino acid sequence as follows:

KTSIKIPSSHNLMKC

(hereafter "EF loop6-Cys").

Murine OPGbp[158-316] having the amino acid sequence from positions 158 to 316 inclusive as set forth in SEQ ID NO:37 of the '315 application and expressed and purified generally as described in Examples 6 and 7, respectively, of the '315 application.

Human OPGbp[159-317] having the sequence from positions 159 to 317 inclusive as set forth in SEQ ID NO:39 of the '315 application and expressed and purified generally as described in Examples 6 and 7, respectively, of the '315 application.

Based on information provided to me, the BB' loop-Cys and EF loop6-Cys peptides were conjugated with keyhole limpet hemocyanin (KLH) prior to immunization.

6. Human OPGbp[159-317] and the KLH-conjugated BB' loop-Cys and EF loop6-Cys peptides were provided to a contract laboratory for immunization of New Zealand white rabbits. Based on information provided to me, serum antibody titers of immunized rabbits were determined by EIA generally as described on p. 48, line 28 to p. 50, line 5 using either human OPGbp[159-317], BB' loop-Cys or EF loop6-Cys peptide coated on microtiter plates.

7. Crude antisera from immunized rabbits were purified by affinity chromatography. Antisera from rabbits immunized with human OPGbp[159-317] were provided to the Genomics Department at Amgen Inc. and purified by applying to an Actigel Ald column coupled to human OPGbp[159-317] and eluting with Pierce Gentle Elution Buffer (Pierce) containing 1% glacial acetic acid. Antisera from rabbits immunized with BB' loop-Cys peptide were provided to the Protein Chemistry Department at Amgen Inc. and purified by applying to a cyanogen bromide-derivatized Sepharose column coupled to murine OPGbp [158-316] and eluting with 0.1M glycine, pH 2.9. In experiments done under my direction, antisera from rabbits immunized with EF loop6-Cys peptide were purified by applying to a Sulfolink column (Pierce) coupled to EF loop-Cys6 peptide and eluting under conditions recommended by the manufacturer. The affinity purified anti-OPGbp antibodies are designated by the OPGbp peptide or polypeptide used for immunization.

8. In experiments done under my direction, the affinity purified anti-OPGbp antibodies were tested for binding

to murine OPGbp[158-316] or human OPGbp[159-317] by EIA generally as described on p. 48, line 28 to p. 50, line 5 in the '315 application and by the following procedure: Each well of a Costar EIA plate (Catalog no. 3590) was coated with 0.1 ml of either a 5 µg/ml human OPGbp[159-317] or a 5 µg/ml murine OPGbp[158-316] solution in Carbonate/Bicarbonate buffer / pH9.6 at 4°C overnight with agitation. After overnight coating, the solution was removed from the plates and 200 µl of 5% BSA blocker (Kirkegaard-Perry (K-P) Labs, Gaithersburg, MD, Catalog no. 50-61-00) at a 1:2 dilution was added to each well and the plates were incubated at room temperature for 1 hour. After incubation, the plates were washed two times with 1X K-P wash solution (Catalog no. 50-63-00). The affinity purified antibodies were serially diluted into PBS (lacking Ca2+ or Mg2+) containing 0.1% Tween-20 (PBS-Tween). 100 µl of each dilution was added to appropriate wells of plates coated with either human OPGbp[159-317] or murine OPGbp[158-316] and incubated for 40 minutes at room temperature with agitation. Following this incubation, the plates were washed three times with the 1X K-P wash solution. To each well of the plate was then added 100 µl of a 1:3000 dilution of Protein A-horseradish peroxidase conjugate (Boehringer Mannheim Biochemicals, Catalog no. 605-295) in PBS-Tween and the plates were incubated 40 minutes further at room temperature with agitation. The plates were then washed four times with the 1X K-P wash solution. Following this final wash, 100 µl of ABTS substrate (K-P Labs, Catalog no. 50-66-01) was added to each well and the absorbance at 405nm was determined at various times using a SPECTRAmax 340 plate reader (Molecular Devices). The results presented in Attachment No. 1 show that the affinity purified anti-OPGbp antibodies bound to both murine and human OPGbp regardless of the OPGbp peptide or polypeptide used for immunization.

9. The affinity purified anti-BB' loop-Cys, anti-EF loop6-Cys, and anti-human OPGbp[159-317] antibodies were provided

to the Analytical Resources Department at Amgen Inc. in order to test for inhibition of osteoclastogenesis *in vitro*. Based upon information provided to me, the mouse bone marrow assay generally as described in Example 8 of the specification was used, with the modification that 20 ng/ml of human OPGbp[159-317], rather than murine OPGbp[158-316], was included. The results provided to me are shown in Attachment No. 2 and indicate that antibodies raised to BB'loop-Cys peptide and human OPGbp[159-317] block osteoclastogenesis whereas antibodies raised to the EF loop6-Cys peptide do not appear to exhibit a significant effect. In the attachment, the A405 values for the anti-human OPGbp and anti-EF loop6-Cys antibodies were normalized to a similar maximum response in order to more readily compare the activities.

10. The affinity purified anti-human OPGbp[159-317] antibodies were provided to the Pathology Department at Amgen Inc. in order to test for effects on bone density. Based on information provided to me, the following *in vivo* assay was used. Male BDF1 mice aged three to four weeks were administered varying doses of affinity purified anti-human OPGbp antibody by daily subcutaneous injection in carrier (PBS/0.1% BSA) starting on day 1. The mice were then x-rayed on day 5. All mice in each treatment group (eight mice per group) and in the PBS/0.1% BSA control group were x-rayed on a single film. The proximal tibial metaphyseal region was compared between pairs of control and treated tibias and scored as a "+" if the treated tibia was denser by visual assessment than the control giving the eight scores shown below. An arbitrary score of 5/8 was required for a "positive" result. The results which were provided to me are shown in Attachment No. 3 and clearly indicate that the anti-human OPGbp increased bone density in this assay at all doses tested.

11. It is clear that the anti-OPGbp antibodies obtained using materials and procedures described in the '315 application block osteoclast formation *in vitro* and promote an increase in bone density *in vivo*. It is apparent that the

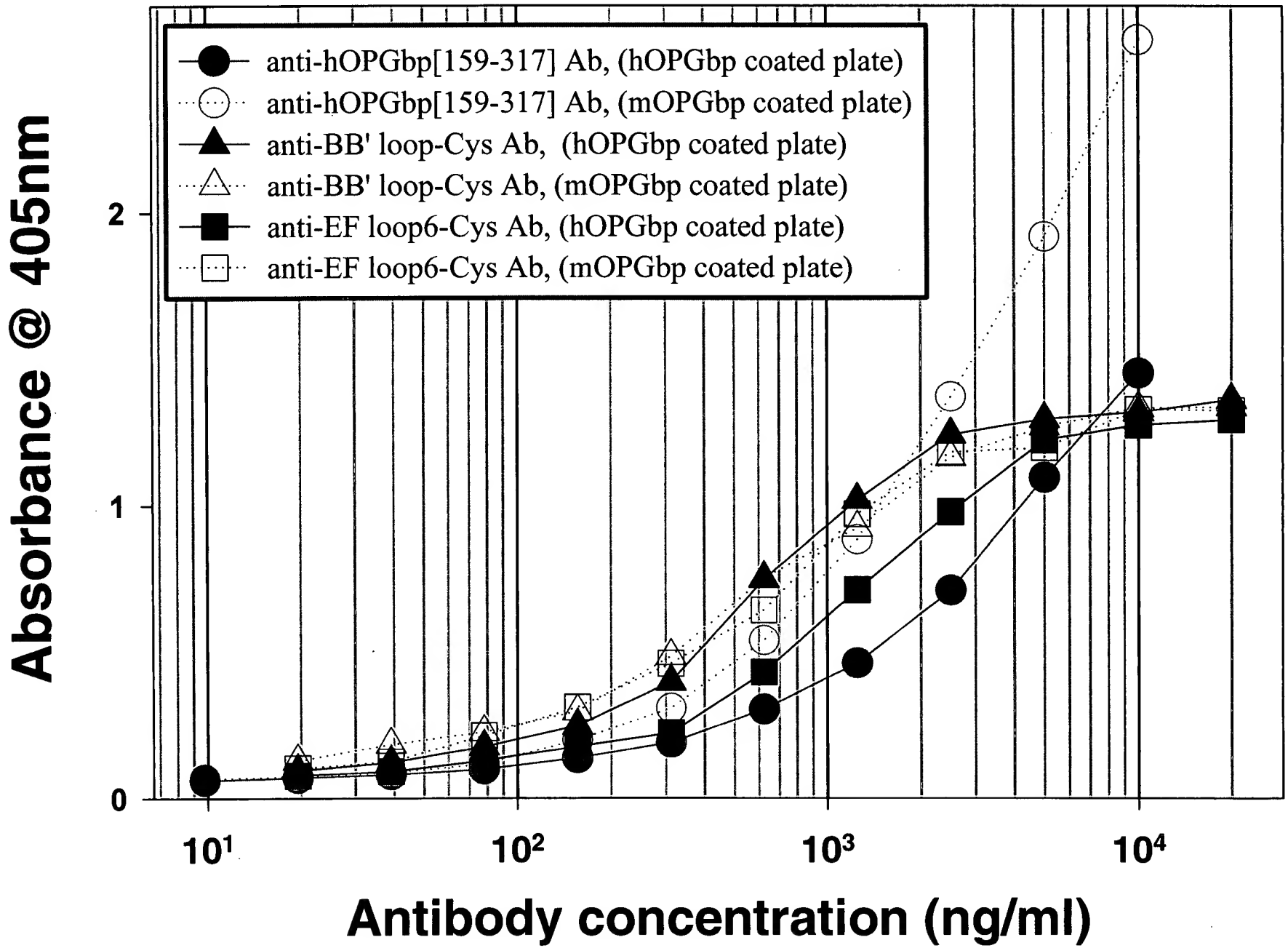
specification enables one skilled in the art without undue experimentation to obtain antibodies which are useful for practicing the methods claimed in the '315 application.

12. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 10 of the United States Code and that such willful false statements may jeopardize the validity of the instant patent application or patent issuing thereon.

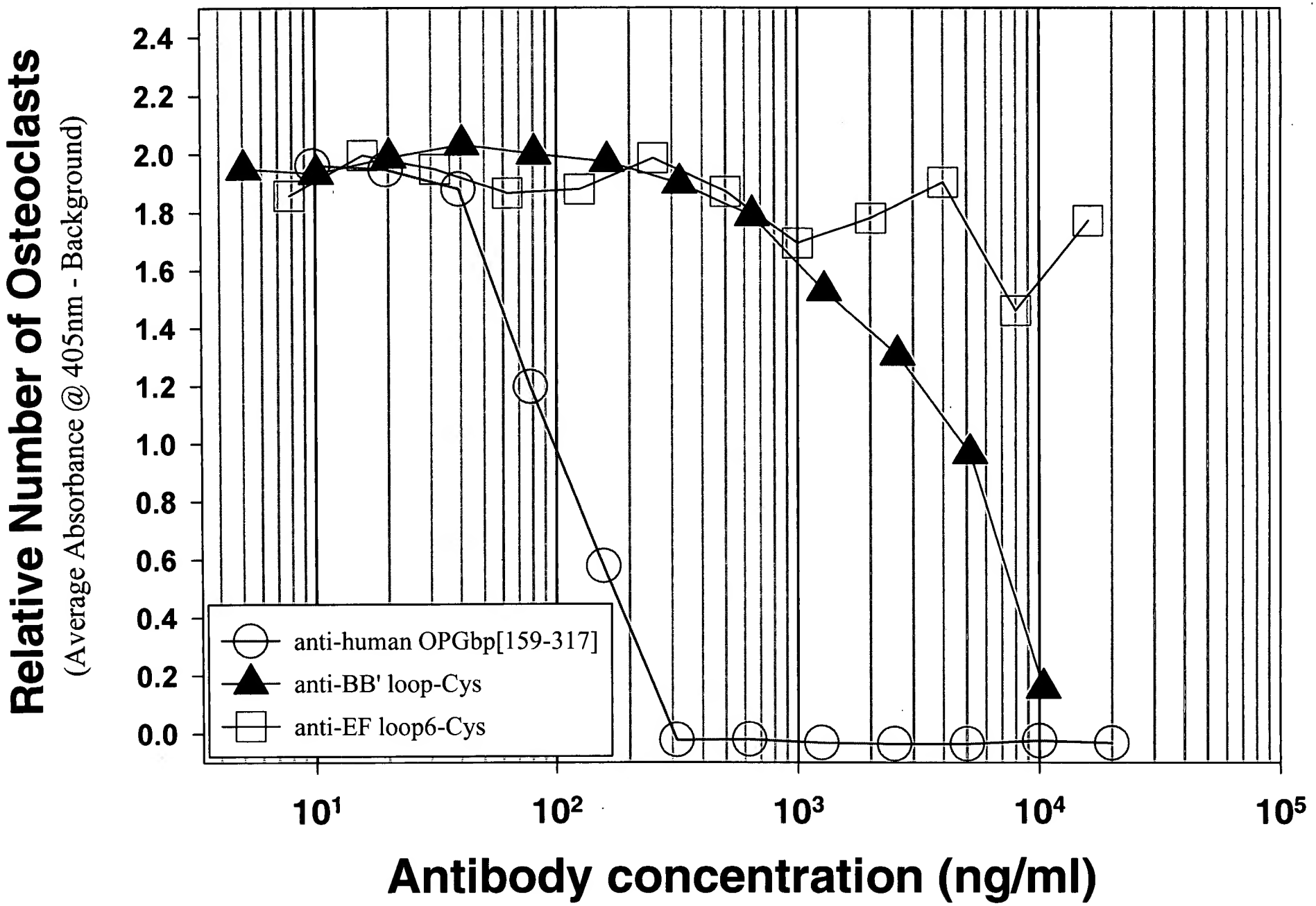
Date: 8-18-2000

John K. Sullivan

ATTACHMENT NO. 1



ATTACHMENT NO. 2



ATTACHMENT NO. 3

Factor	Dose	1	2	3	4	5	6	7	8	Result	
Anti-human OPGbp antibody	10 mg/kg	+	+	+	+	+	+	+	+	Positive	8/8
Anti-human OPGbp antibody	3.0 mg/kg	+	+	+	+	+	+	+	+	Positive	8/8
Anti-human OPGbp antibody	1.0 mg/kg	+	+	+	+	+	+	+	+	Positive	8/8
Control (PBS/0.1% BSA)		-	-	-	-	-	-	-	-	Negative	0/8

Exhibit A

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Characterization of Mouse Monoclonal Antibodies to Human Interferon-Gamma

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Abstract Mouse monoclonal antibodies (mAb) to human interferon-gamma (HuIFN-γ) were characterized. The mAbs studied—E4-18, G4-15, and SAT-1—which are all IgG1-type, reacted to all HuIFN-γ molecular species, both glycosylated and non-glycosylated. Affinity constants calculated of E4-18 and G4-15 didn't have considerable differences for both kinds of HuIFN-γ ($1–3 \times 10^8$ liter/mol), but SAT-1 had a difference—a higher value (10^{10} liter/mol) for the former than for the latter (8×10^8 liter/mol). In epitope specificity, the results suggested that E4-18 and G4-15 recognized an overlapped region remote from the region of SAT-1. Competition experiment using synthetic peptides suggested that epitope of G4-15 is around N9-26 of the HuIFN-γ sequence. Those mAbs could be used for sandwich radioimmunoassay of HuIFN-γ using double mAbs in two combinations, one (G4-15/E4-18) based on dimer forms of HuIFN-γ and the other (SAT-1/E4-18) based on epitope difference. The mAbs are all neutralizing antibodies in which SAT-1 neutralized at a lower concentration than did G4-15, and at a much lower one than did E4-18. The receptor binding of HuIFN-γ was inhibited by mAbs G4-15 and SAT-1. Efficacy of G4-15 and SAT-1 for the inhibition correspond with that for neutralization.

IFN-γ has various effects on cells especially immune cells as an important lymphokine (28). Through recombinant technology, the primary structure of HuIFN-γ has been elucidated (6), and its correspondence to the structure of natural HuIFN-γ (nHuIFN-γ) molecules has also been determined (22). Thanks to the massive production of recombinant HuIFN-γ (rHuIFN-γ), much progress has been made, but many problems remain to be solved, especially because of its pleiotropic effects on cells (21, 28).

Until now, mouse mAbs to HuIFN-γ were effectively utilized for the purification of nHuIFN-γ (10, 16, 19), sensitive IFN assay (3), identification of HuIFN-γ (18, 23, 27), etc. In this report, we describe the characteristics of mAbs to HuIFN-γ,

two of which E4-18 and G4-15, were produced by Miyata et al (16), and one, SAT-1 was produced by Tsukui (unpublished).

HuIFN-γ molecules were shown to have a molecular weight (M.W.) of 25,000, 20,000 and 15,000 molecular species for nHuIFN-γ (9, 16, 22, 29), and 17,000 for rHuIFN-γ (2), and 21,000 to 25,000 for rHuIFN-γ produced in animal cells (4, 5) as determined by SDS/polyacrylamide gel electrophoresis (SDS/PAGE), and those molecules exist as dimers in neutral solution (2, 4, 16, 29). Our characterization of mAb was performed on those HuIFN-γs upon considering their characteristics, glycosylated or non-glycosylated, and dimer forms.

MATERIALS AND METHODS

HuIFN preparations. Natural HuIFN-γ was produced in culture fluids of human leukocytes stimulated by concanavalin A (Con A). The details of the procedure have been described elsewhere (14). Partially purified nHuIFN-γ preparations ($0.3–2 \times 10^6$ IU/mg protein) which were processed by Phenyl-Sepharose, silicic acid, Con A-Sepharose, CM Toyo Pearl 650S ion-exchange columns, and gel filtration on a BioGel P100 column, were used for immunogen and screening of mAbs to HuIFN-γ. Purified nHuIFN-γ preparations ($2–4 \times 10^7$ IU/mg protein) purified by E4-18 antibody column (16) were used for the characterization of the mAbs. Recombinant HuIFN-γ was produced in $F_\gamma 56$ cells (rHuIFN-γ ($F_\gamma 56$)) which are mouse C127 cells transformed with a chimeric plasmid consisting of bovine papilloma virus and human interferon-gene (5). The IFN was purified by E4-18 antibody column. Purified preparations had $2–4 \times 10^7$ IU/mg protein of specific activity, which consisted of a protein band at a M.W. of 23,000 when analyzed by SDS/PAGE. Recombinant HuIFN-γ produced in *E. coli* (rHuIFN-γ) ($0.4–1 \times 10^7$ IU/mg protein) was supplied by Shionogi Seiyaku, Co., rHuIFN-α2 (5×10^8 IU/mg protein) by Schering Corporation through Nippon Essex, and rHuIFN-β (1×10^7 IU/mg protein) by Toray Industries, Inc.

^{125}I-labeling of HuIFN-γs. HuIFN-γs were all reacted with ^{125}I-Bolton and Hunter reagent (BH: New England Nuclear; 2,000 Ci/mmol) in 0.1 M borate buffer, pH 8.0 at 4 C for 0.5 to 1 hr. Labeled IFNs were obtained in the void fraction of BioGel P-6DG (Bio-Rad) and stored at 4 C in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA), antibiotics mixture (ABM: penicillin G and streptomycin sulfate), and gentamicin sulfate.

Monoclonal antibodies to HuIFN-γ. Monoclonal antibodies to HuIFN-γ, E4-18 and G4-15 were produced by fusion of spleen cells from immune mice with mouse myeloma cells, X63-Ag8-653 as described by Oi and Herzenberg (20). Hybridoma cells were screened by a direct binding assay. Partially purified nHuIFN-γ was used as immunogen and for the screening of the mAbs. For SAT-1, partially purified HuIFN-γ produced by a human T-lymphoblastoid cell line, TCL-Fuj (15), was used.

Purification of mAbs to HuIFN-γ. For the purification of E4-18 and G4-15 mAbs, pristane-primed BALB/c mice were injected with $0.5–2 \times 10^7$ hybridoma cells, and

ascitic fluids were withdrawn, and fractionated by precipitation with ammonium sulfate at 50% saturation. The precipitate was dissolved in PBS, and dialyzed against 10 mM sodium phosphate buffer (Na-P), pH 8.0 containing 0.15 M NaCl. The dialyzate was clarified by centrifugation and applied to a protein A-Sepharose (Pharmacia) column equilibrated with the same buffer. Immunoglobulin was eluted with 0.1 M sodium citrate buffer, pH 5.0. SAT-1 mAb was obtained from ascitic fluids of BALB/c nu/nu mice, fractionated by precipitation with ammonium sulfate at 50% saturation. The precipitate was dissolved in 20 mM Na-P, pH 7.2, dialyzed and loaded on a DEAE-Affigel blue (Bio-Rad) column equilibrated with the buffer. The column was then washed with the buffer but containing 25 mM NaCl, and eluted with the buffer containing 75 mM NaCl (1). The eluate was dialyzed against PBS, after concentration by Aquacide II (Calbiochem).

^{125}I-labeled G4-15 and SAT-1 IgGs. G4-15 mAb (20 μg IgG in 20 μl) or SAT-1 mAb (8.5 μg IgG in 10 μl), was reacted with 334 μCi of ^{125}I-BH reagent (2,000 Ci/mmol) at 0 C for 2 hr (G4-15), or 3 hr (SAT-1) in 0.1 M borate buffer, pH 8.2. Thus ^{125}I-labeled G4-15 IgG (initial activity: 8.26×10^6 cpm/μg protein) and ^{125}I-labeled SAT-1 IgG (2.00×10^7 cpm/μg protein as above) were obtained.

Immunoglobulin isotype determination. Subtype analysis by Ouchterlony immunodiffusion showed IgG1 for both E4-18 and G4-15 mAbs. In the case of SAT-1 mAb, IgG1 (K) was verified by enzyme immunoassay.

Assay of IFN. The antiviral activity of IFN was assayed by inhibition of the cytopathic effect of Sindbis virus in human FL cells (FL_{5-1}) as described (16). In most cases, the titers of HuIFN-γ were expressed in International IFN unit (IU) by calibrating against the HuIFN-γ reference standard Gg23-901-530 provided by the National Institute of Health (Bethesda, Md., U.S.A.). One experimental unit (1 EU: 50% inhibition of cytopathic effect) corresponds to 0.2 to 0.3 IU.

Immunoprecipitation with pansorbin. ^{125}I-labeled HuIFN-γ and mAb (purified IgG) were mixed in 200 μl/tube of 10 mM tris-HCl buffer, pH 8.1, containing 0.1 mM EDTA, 0.2% BSA (NET). After incubation at 37 C for 2 hr, followed by addition of RαMIgG (rabbit anti-mouse immunoglobulin, 0.2 μg/20 μl/tube), 30 μl/tube of 10% (v/v) pansorbin (*Staphylococcus aureus* cells; Calbiochem) in NET containing 0.05% (w/v) Nonidet P40 (NP40; Bethesda Research Laboratories) (NP40/NET) was added. Incubation was continued at 37 C for 1 hr, followed by 4 C overnight. The immunoprecipitate were collected by microcentrifugation (Beckmann Microfuge 12) and washed twice with NP40/NET. Radioactivity was counted in a gamma counter.

Sandwich radioimmunoassay using double mAbs. Flexible plates (Falcon 3612) were coated with 1.5 μg/50 μl/well of mAb (purified IgG) in PBS containing 0.02% NaN_3 and kept at 4 C overnight. The wells were washed with PBS, and 150 μl/well of PBS containing 3% BSA was added. After incubation at 37 C for 30 min, the block solution was removed and the wells were washed twice with PBS, HuIFN-γ samples in 100 μl/well of PBS containing 0.1% BSA and ABM were added. After incubation at 37 C for 1 to 2 hr, the wells were washed three times with PBS, then 50 μl/well of ^{125}I-labeled G4-15 or SAT-1 IgG in MEM (Nissui Co.) containing 3%

newborn calf serum and 0.1% glucose (growth medium for FL cells: FLGM) was added, and incubation was continued at 37 C for 2 hr. The wells were washed three times with PBS containing 0.02% (w/v) Tween 20 (PBS/Tween), cut, and the radioactivities were counted in a gamma counter.

Solid-phase binding assay using RαMIgG. Flexible plates (Falcon 3612) were coated with RαMIgG (10 μg/well) in 100 μl of 5 mM glycine buffer, pH 9.2, containing 0.02% NaN_3, and kept at 4 C overnight. After washing with PBS/Tween, mAb in 100 μl/well of FLGM was added and the solution was incubated at 37 C for 1 hr. The plates were washed three times with PBS, then ^{125}I-labeled HuIFN-γ in 100 μl/well of PBS containing 0.2% BSA and ABM was added. The plates were incubated at 37 C for 2 hr, then washed three times with PBS/Tween. The wells were cut and the radioactivities were counted in a gamma counter.

^{125}I-labeled HuIFN-γ binding to FL cell receptor. FL_{5-1} cells were seeded in 1 ml/well of FLGM on 24-well plates and incubated at 37 C overnight in humidified air containing 5% CO_2. The monolayers (5×10^5 cells/well) were then washed once with cold FLGM on ice, 0.4 ml/well of ^{125}I-labeled HuIFN-γ in FLGM containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and gentamicin sulfate (50 μg/ml) were added, and the plates were incubated at 22 C as indicated. Wells were washed three times with cold PBS, and the cells were solubilized in 1 ml/well of 1% SDS at 37 C for 10 min and the radioactivities were counted in a gamma counter. Specific binding was measured in the presence of excess unlabeled HuIFN-γ.

Protein assay. The protein content was determined by a micro-method using Coomassie brilliant blue G250 (Eastman Kodak Co.) with BSA as a standard (26).

RESULTS AND DISCUSSION

Reactivity of mAb to Various HuIFNs Characterized in Liquid-Phase System

Firstly, it was ascertained that mAbs, E4-18, G4-15, and SAT-1, all reacted with HuIFN-γs, nHuIFN-γ, rHuIFN-γ, and glycosylated rHuIFN-γ (rHuIFN-γ (F_r56)), but not with HuIFN-α and HuIFN-β.

Experiments were done with incubation of ^{125}I-labeled HuIFN-γ in solution and immune complex was precipitated by pansorbin. In this system, the three mAbs reacted with the above three kinds of ^{125}I-labeled HuIFN-γ at effective IgG doses respectively. The result of competitive radioimmunoassay using ^{125}I-labeled rHuIFN-γ (F_r56) as a tracer is shown in Table 1. The competitor concentration (unlabeled HuIFN-γs) giving 50% inhibition of tracer-antibody binding showed the sensitivity of this system. No displacement was seen with other types of HuIFNs (rHuIFN-α2 and rHuIFN-β, 1.5×10^5 IU/ml each) with all three mAbs in this system.

The antibody affinity constant (K) was calculated from the molar competitor concentration giving 50% inhibition of tracer-antibody binding in the competitive radioimmunoassay, the molar tracer concentration in the assay, and the amount of tracer bound in the absence of competitor by following the method of Müller (17).

Table 1. Calculation of K from data obtained by competitive radioimmunoassay (RIA)

mAb	Tracer	Competitor	$-[I_t]^{a}$ (IU/ml)	$-[I_t]^{a}$ (M)	$-[T_t]^{b}$ (M)	$K = 8/3([I_t] - [T_t])$ (liter/mol)
E4-18	rHuIFN-γ ($F_2$56)	rHuIFN-γ ($F_2$56)	4,000	8.7×10^{-9}	2.0×10^{-10}	3.2×10^{8}
E4-18	rHuIFN-γ ($F_2$56)	rHuIFN-γ	4,000	2.4×10^{-8}	2.0×10^{-10}	1.1×10^{8}
G4-15	rHuIFN-γ ($F_2$56)	rHuIFN-γ ($F_2$56)	4,000	8.7×10^{-9}	2.0×10^{-10}	3.2×10^{8}
G4-15	rHuIFN-γ ($F_2$56)	rHuIFN-γ	3,500	2.1×10^{-8}	2.0×10^{-10}	1.3×10^{8}
SAT-1	rHuIFN-γ ($F_2$56)	rHuIFN-γ ($F_2$56)	200	4.0×10^{-10}	2.0×10^{-10}	1.3×10^{10}
SAT-1	rHuIFN-γ ($F_2$56)	rHuIFN-γ	580	3.4×10^{-9}	2.0×10^{-10}	8.3×10^{8}

a) $[I_t]$, the competitor concentrations giving 50% inhibition of tracer-antibody binding in the RIA. The molar concentrations of HuIFN-γs were estimated as the specific activities, 2×10^{7} IU/mg protein (rHuIFN-γ ($F_2$56)) and 10^{7} IU/mg protein (rHuIFN-γ) and assuming the molecular weight of the former, 23,000 and of the latter, 17,000.

b) $[T_t]$, the total tracer concentrations, the specific activity of ^{125}I-labeled rHuIFN-γ ($F_2$56), 508 cpm/IU; K, antibody affinity constant was calculated by the method of Müller (17).

Table 2. The binding inhibition of ^{125}I-labeled HuIFN-γ by mAbs on mAb-coated plates

mAb in incubation mixture	Bound[a]			
	Plates coated with mAb			
	E4-18	G4-15	SAT-1	No
no	5,558 ± 250	2,136 ± 359	2,279 ± 39	208 ± 33
E4-18	2,175 ± 734	426 ± 21	1,597 ± 264	—
G4-15	232 ± 49	155 ± 17	1,136 ± 53	—
SAT-1	4,431 ± 502	1,120 ± 46	139 ± 17	—

[a] Mean cpm ± standard deviations (triplicate). The coating of mAb and blocking procedure are the same as in Fig. 1. 1.06×10^4 cpm of ^{125}I-labeled HuIFN-γ (F_γ56) (600 cpm/IU) and mAbs (1 μg IgG/well) were preincubated at 37 C for 1 hr in 100 μl/well of FLGM on 96-well plates, transferred to pre-coated flexible plates, and incubated at 37 C for 2 hr. The plates were washed three times with PBS/Tween, cut, and the radioactivities were determined by a gamma counter.

The K values were for competitors, rHuIFN-γ (F_γ56) and rHuIFN-γ. As seen in Table 1, three mAbs are relatively high affinity antibodies, SAT-1 having a higher K value than E4-18 and G4-15. The difference of the K values between both kinds of HuIFNs, glycosylated (rHuIFN-γ (F_γ56)) and non-glycosylated (rHuIFN-γ), was not significant with E4-18 and G4-15 mAbs, but it was fairly significant with SAT-1 mAb, which had a higher value to the glycosylated than to the non-glycosylated one. It shows that the epitope region of SAT-1 mAb is in a peptide part, and is more responsive to glycosylated form of HuIFN-γ. This would probably result from a difference in tertiary structure of these two. Therefore it is possible that the affinity of SAT-1 changes in corresponding to the degree or a difference of glycosylation of HuIFN-γs which are produced in different cells.

The Epitope Difference between Three mAbs

To characterize a difference in epitope specificity among mAbs, the binding inhibition by one mAb of ^{125}I-labeled HuIFN-γ was examined on the other mAb-coated plates. On coated wells, the preincubation mixtures of ^{125}I-labeled rHuIFN-γ (F_γ56) with or without excess molar concentration of the mAbs were added, and further incubated. As shown in Table 2, when the same mAbs were employed the amounts of labeled HuIFN-γ decreased to the level of the non-specific binding (no coating with mAb) in the cases of G4-15 and SAT-1. In the case of E4-18, however, binding inhibition was unexpectedly weak; the reason is not clear, but it may somehow result from the characteristics of this mAb in solution on the mAb-coated plates. Setting this aside, in the case of E4-18 coated/G4-15 mixed, and G4-15 coated/E4-18 mixed, the amounts of labeled HuIFN-γ decreased almost to the same level as that of non-specific binding to indicate that both mAbs, E4-18 and G4-15, recognize an overlapped region of HuIFN-γ, which is remote from a region of SAT-1. Moderate binding inhibition was also observed in the case of G4-15 coated/SAT-1 mixed, SAT-1 coated/E4-18 mixed, and SAT-1 coated/G4-15 mixed; this may indicate a slight overlap in their epitope regions, but if considered

Table 3. Binding of ^{125}I-labeled HuIFN-γ to G4-15 mAb: Competition by synthetic peptides of HuIFN-γ[a]

Competitors Conc. (μM)	Bound[b]
None	1,385 ± 28
nHuIFN-γ, 1×10^4 IU/ml (0.02)	244 ± 16
3×10^4 IU/ml (0.06)	189 ± 30
1×10^5 IU/ml (0.20)	100 ± 18
Synthetic peptides	
9–26, 5 μg/ml (2.2)	696 ± 49
16–27, 5 μg/ml (3.3)	1,343 ± 6
40–47, 5 μg/ml (4.4)	1,229 ± 66
84–95, 5 μg/ml (3.2)	1,204 ± 67
None/no mAb	270 ± 130

[a] Experiment was performed with ^{125}I-labeled nHuIFN-γ (3.6×10^4 cpm/36 IU/100 μl/well, 1.1×10^7 cpm/μg protein), unlabeled nHuIFN-γ (2×10^7 IU/mg protein), and G4-15 IgG (0.3 μg/ml) in a solid-phase binding assay on plates coated with RμMIgG. Details are described in "MATERIALS AND METHODS." Maximum binding was 2,083 cpm with 10 μg/ml of G4-15 IgG.

[b] Mean cpm ± standard deviations (duplicate).

as a difference of the coating efficiency of the mAb (described below in "Sandwich radioimmunoassay"), the results would not be sufficient to prove it. And it would rather likely come from higher affinity of SAT-1 mAb than the other two.

Demonstration of the Epitope of G4-15 mAb

To determine the epitope region of the mAbs, competitive binding assay by the synthetic peptides was performed in solid-phase system using ^{125}I-labeled HuIFN-γ and unlabeled mAb on RαMIgG-coated plates. The four synthetic peptides used were prepared by Shimizu who took into account the hydrophilic regions of the cDNA-predicted sequence of HuIFN-γ (6) to be intrinsic for antigenicity (7). As shown in Table 3, the binding of ^{125}I-labeled nHuIFN-γ to G4-15 mAb was inhibited by synthetic peptides N9-26, but not by N16-27, N40-47, or N84-95. An approximately 100-fold, or more, excess concentration of the peptide was required to inhibit the binding as compared to that of unlabeled HuIFN-γ. The results suggest that the epitope of G4-15 mAb is in the N-terminal region around N9-26. Russell et al (24) reported that the N-terminal end of MuIFN-γ is an important antigenic region. A similar experiment was performed with SAT-1 mAb, but no significant inhibition was observed.

Sandwich Radioimmunoassay of HuIFN-γ Using Double mAbs

Sandwich radioimmunoassay of HuIFN-γ was examined in making use of the mAbs. HuIFN-γ samples were sandwiched with ^{125}I-labeled mAb on the plates precoated with another mAb.

The combination of ^{125}I-labeled G4-15 IgG and E4-18 IgG coating could be used, but the sensitivity was not high, as shown in Fig. 1a. Since the epitope

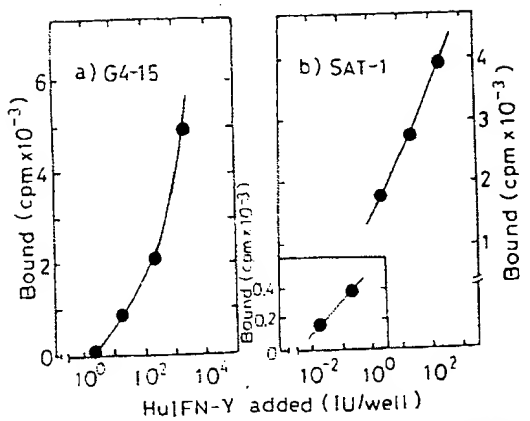

Fig. 1. Sandwich radioimmunoassay using double mAbs. a) HuIFN-γ samples (rHuIFN-γ (F_r56)) were placed in wells coated with E4-18 IgG. ^{125}I-labeled G4-15 IgG (8.5×10^4 cpm/18 ng/100 μl/well) was added. b) On E4-18-coated wells, ^{125}I-labeled SAT-1 IgG (2.1×10^4 cpm/1.3 ng/50 μl/well) was added. The experimental procedure is described in "MATERIALS AND METHODS." Each point represents the mean cpm of duplicate determinations.

regions of those mAbs are overlapped and HuIFN-γ molecules exist in dimer forms in neutral solution, it must be that the binding of ^{125}I-labeled G4-15 IgG on E4-18-coated plates is based on the dimer form of HuIFN-γ as reported by Le et al (12). Another combination, SAT-1 IgG as a tracer and E4-18 coating, showed a much higher sensitivity as shown in Fig. 1b. The lower detection limit was 100 IU/ml of HuIFN-γ (F_r56) in the former combination, and 0.4 IU/ml in the latter. The binding of ^{125}I-labeled rHuIFN-γ on E4-18-coated well was found to have a plateau at a concentration of 3 μg/ml (70 ng/well retained). The combination of SAT-1/E4-18 can be used as a sensitive and accurate assay.

With the other combination, that is, ^{125}I-labeled SAT-1 IgG and SAT-1 IgG coating, there was no binding at all, as was also the case with the combination of ^{125}I-labeled G4-15 IgG and SAT-1 coating. Even if two mAbs had a difference in epitope specificity, or HuIFN-γ molecules existed as dimers (or oligomers), the sandwich radioimmunoassay does not necessarily work as it depends on the configuration of mAbs. SAT-1 is suitable for using as a tracer but not for coating.

Neutralization of Antiviral Activity

The three mAbs all neutralize the antiviral activity of HuIFN-γ. Their neutralization effect was examined by adding a fixed amount of the IgGs at several concentrations to serial 2-fold dilutions of nHuIFN-γ. Complete neutralization was achieved with all three mAbs, but at different IgG doses. SAT-1 neutralized at a lower concentration than did G4-15 and at a much lower dose than did E4-18, as shown in Table 4. The SAT-1 IgG concentration required to reduce the IFN titer to one sixty-fourth was approximately 80-fold the molar excess of antibody to

Table 4. Neutralization by mAbs of antiviral activity of HuIFN-γ[a]

mAbs IgG conc. (μg/ml)	IFN neutralized (N)[b]		
	E4-18	G4-15	SAT-1
100	80	—	—
25	20	64	—
10	8	32	—
2.5	2.5	8	—
1	—	4	320
0.25	—	—	64

[a] Serial 2-fold dilutions of nHuIFN-γ samples were mixed with mAbs at the several concentrations indicated in 100 μl/well of FLGM and incubated at 37 C for 1 hr. Samples (in duplicate) were transferred to FL monolayers on microplates, incubated at 37 C overnight, and processed by IFN assay.

[b] N, IFN concentration (EU/ml) reduced to 1 EU/ml by antibody (8).

HuIFN-γ, if assuming molecular weight of nHuIFN-γ, 25,000, and its specific activity, 4×10^7 IU/mg protein.

With respect to neutralization of mAbs against natural or recombinant Hu-IFN-γs, Liang et al (13) reported that mAbs BG1 to BG5 reacted more effectively against rHuIFN-γ rather than nHuIFN-γ; the reverse was true with a mAb named GIF1 reported by Le et al (11); and a mAb named B3 was reported by Le et al (10) to react equally against both HuIFN-γs. In the present study, the results (Table 1) show SAT-1 mAb reacts more effectively to glycosylated than to non-glycosylated HuIFN-γ, and the difference is not marked with E4-18 and G4-15 mAbs. Therefore efficacy of SAT-1 for neutralization may be changed with the difference of glycosylation of HuIFN-γ molecules.

Preincubation of mAb and HuIFN-γ seemed to be somewhat effective, that is, in one experiment using G4-15 mAb, there was observed a certain difference in neutralization with or without preincubation.

^{125}I-Labeled HuIFN-γ Binding to FL Cells: Inhibition of the Binding by mAbs G4-15 and SAT-1

Before testing the mAbs inhibition of the receptor binding of HuIFN-γ, we examined the binding of ^{125}I-labeled rHuIFN-γ to FL cells. The specific binding at 22 C reached a plateau at 3 hr (data not shown). The specific binding for 3 hr is shown in Fig. 2. The Scatchard plot (25) of this data is shown in the inset. Kd was estimated to be 2.45×10^{-9} M with 99,600 binding sites/cell.

When ^{125}I-labeled rHuIFN-γ (F_r56) was preincubated with each mAb, and FL cells were added, the binding was inhibited by both G4-15 and SAT-1 mAbs, as shown in Fig. 3. The half maximum inhibitory doses of the mAbs (ID_{50}) estimated were 13.3 nM (G4-15) and 0.23 nM (SAT-1). The ID_{50} of SAT-1 mAb was nearly the same order of the concentration of HuIFN-γ in the incubation mixture. Neutralization of antiviral activity by SAT-1 must result from its effective inhibition of the binding of HuIFN-γ to receptor sites. The ID_{50} for G4-15 mAb

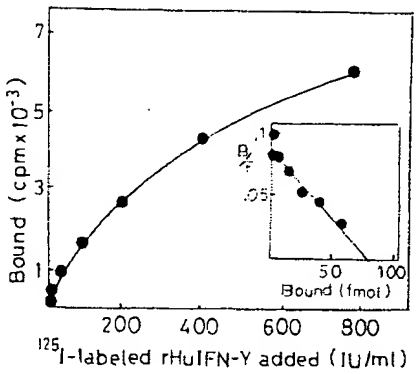

Fig. 2. ^{125}I-labeled HuIFN-γ binding to FL receptors. Confluent FL monolayers (24-well plates) were incubated with increasing concentrations of ^{125}I-labeled rHuIFN-γ (0.6×10^7 cpm/μg protein, 608 cpm/IU) in 0.4 ml/well of incubation medium with or without 170-fold excess of unlabeled rHuIFN-γ at 22 C for 3 hr. The experimental procedure is described in "MATERIALS AND METHODS." Each point represents the mean cpm of triplicate determinations (standard deviations of the points averaged 5.9%). Non-specific binding was subtracted (24% average). The inset shows Scatchard plots of this data.

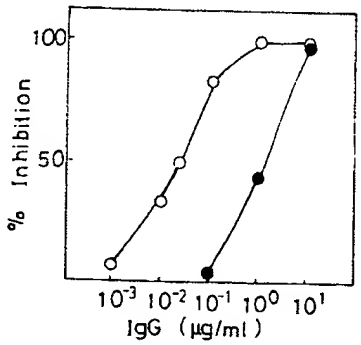

Fig. 3. Receptor binding inhibition of ^{125}I-labeled HuIFN-γ by G4-15 or SAT-1 mAb. ^{125}I-labeled rHuIFN-γ ($F_7$56) samples (3×10^6 cpm/μg protein; 613 cpm/IU) were pre-incubated with mAbs, G4-15 (●) or SAT-1 (○) in indicated concentrations at 37 C for 1 hr. The mixtures (2.5×10^4 cpm/41 IU/0.4 ml/well) were added to FL cells and incubated at 22 C for 4.5 hr. Each point represents the mean cpm of duplicate or triplicate (no mAb) determinations. The average standard deviation for all the values was 6.5% of the counts. Non-specific binding (determined by addition of unlabeled HuIFN-γ) was subtracted.

was about 60 times that for SAT-1 mAb, and neutralization by the former required about a 100-fold higher concentration than by the latter. Thus, the efficacy of G4-15 and SAT-1 mAbs for receptor binding inhibition corresponds respectively to that for neutralization.

If the best use is made of their different characteristics as described here, the three mAbs will be useful in analysis of various effects of HuIFN-γ on cells and of the structure-function relationships.

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Exhibit B

THE MOUSE/HUMAN CHIMERIC MONOCLONAL ANTIBODY cA2 NEUTRALIZES TNF IN VITRO AND PROTECTS TRANSGENIC MICE FROM CACHEXIA AND TNF LETHALITY IN VIVO

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The pleiotropic cytokine tumour necrosis factor-α (TNF) is thought to play a central role in infectious, inflammatory and autoimmune diseases. Critical to the understanding and management of TNF-associated pathology is the development of highly specific agents capable of modifying TNF activity. We evaluated the ability of a high affinity mouse/human chimeric anti-TNF monoclonal antibody (cA2) to neutralize the in vitro and in vivo biological effects of TNF. cA2 inhibited TNF-induced mitogenesis and IL-6 secretion by human fibroblasts, TNF-priming of human neutrophils, and the stimulation of human umbilical vein endothelial cells by TNF as measured by the expression of E-selectin, ICAM-1 and procoagulant activity. cA2 also specifically blocked TNF-induced adherence of human neutrophils to an endothelial cell monolayer. Receptor binding studies suggested that neutralization resulted from cA2 blocking of TNF binding to both p55 and p75 TNF receptors on the cells. In vivo, repeated administration of cA2 to transgenic mice that constitutively express human TNF reversed the cachectic phenotype and prevented subsequent mortality. These results demonstrated that cA2 effectively neutralized a broad range of TNF biological activities both in vitro and in vivo.

Tumour necrosis factor-α (TNF) is a cytokine that exhibits a pleiotropic spectrum of activities, with receptors found on virtually all cell types examined.[1,2] The natural functions of TNF are thought to include modulation of the host immune and inflammatory response to a variety of infectious, malignant and autoimmune conditions as part of a complex regulatory mechanism in which numerous other cytokines participate.[3] While initial TNF expression in response to infection or injury would be considered beneficial, excessive production, usually by activated monocytes and macrophages, can result in significant pathological changes.

TNF has been implicated as the primary mediator in bacterial sepsis since it is the first proinflammatory cytokine detected in primate and human volunteer studies where serum cytokine levels were measured after administration of endotoxin.[4] Administration of TNF to rodents and dogs induced a profile of pathophysiological changes and lethality similar to that seen after endotoxin challenge.[5,6] Neutralizing antibodies to TNF have been shown to prevent physiological changes and death in animal models of endotoxin and bacteremia.[7-9]

There are also a number of autoimmune disorders in which TNF appears to play a significant role,[10-12] but the evidence is most persuasive in rheumatoid arthritis.[13-16] Rheumatoid arthritis (RA) is characterized by a chronic inflammation of the synovial lining of multiple joints. Synovial cells proliferate along with infiltrating inflammatory cells and vascularity increases markedly. Ultimately, the release of degradative enzymes results in irreversible erosion of the bone and cartilage components of the joint.[13,14] Levels of TNF are not only elevated in synovial fluid from the joints of RA patients[17,18] but cells from the synovial fluid continue to produce TNF when cultured in vitro.[19] Perhaps the most direct evidence that TNF plays a pivotal role in the development of arthritis was obtained by the constitutive expression of TNF in transgenic mice.[20] Such mice develop chronic inflammatory polyarthritis at a specific age (depending on the transgenic mouse line)

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and with a 100% phenotypic penetrance. Thus, TNF appears to be an attractive target and an antibody which efficiently neutralizes human TNF may be an effective treatment in RA.[20,21]

We have previously described the construction of a chimeric mouse/human IgG_1 monoclonal antibody which binds to TNF.[22] This antibody, designated cA2, exhibited high affinity and specificity for TNF and neutralized both recombinant and natural human TNF in the standard assay of TNF cytotoxicity. cA2 was also shown to be highly species-specific, neutralizing TNF from only humans and chimpanzees. The safety and potential efficacy of cA2 in treating autoimmune disorders is currently being evaluated in human clinical trials.[23,24]

In this study we examined the effect of cA2 on the in vitro biological activity of TNF and on TNF-receptor interactions. The ability of cA2 to modulate the in vivo activity of TNF was also investigated using an established transgenic mouse line that develops a cachectic phenotype and accelerated mortality due to constitutive expression of human TNF.[25]

RESULTS

Effect of cA2 on TNF-stimulated FS-4 fibroblasts

The fibroblast cell line FS-4 proliferates and secretes IL-6 in response to recombinant human TNF-α (TNF).[26,27] As shown in Table 1, TNF at concentrations ranging from 0.3 to 7.5 ng/mL induced FS-4 fibroblasts to produce IL-6 levels ranging from 1300 to 2500 pg/mL. When cA2 was added to the medium at the same time as TNF, the induction of IL-6 was completely blocked at the 0.3 and 1.5 ng/mL TNF dose levels, and only a small amount of IL-6 was detected (300 pg/mL) at the highest (7.5 ng/mL) TNF dose. The control antibody had no effect.

Similarly, cA2 was shown to block the mitogenic effect of TNF on FS-4 fibroblasts (Table 2). When cA2 was added to the culture medium with TNF, proliferation was blocked at all three TNF levels tested (0.1, 0.5 and 2 ng/mL). The control IgG had no effect on the TNF-induced proliferation of FS-4 fibroblasts. A significant difference ($P \leq 0.03$ at all concentrations) was demonstrated between the cA2 and control groups using the nonparametric Wilcoxon test. In additional experiments, cA2 also completely inhibited mitogenesis at TNF concentrations up to 8 ng/mL (data not shown).

Effect of cA2 on TNF-stimulated human umbilical vein endothelial (HUVE) cells

HUVE cells produce a procoagulant activity (PCA) when exposed to TNF which appears to be related to

TABLE 1. Neutralization of TNF-induced IL-6 secretion by fibroblasts

	IL-6 production (pg/mL)			
Antibody	No TNF	0.3 ng/mL TNF	1.5 ng/mL TNF	7.5 ng/mL TNF
None	<200	1360	2000	2560
Control	<200	1600	1960	2160
cA2	<200	<200	<200	300

Recombinant human TNF, preincubated with or without 4 μg/mL cA2 or control antibody, was added to cultures of FS-4 human fibroblasts. After 18 h incubation, IL-6 levels in the supernatant were determined by immunoassay.

TABLE 2. Neutralization of TNF-induced mitogenesis in fibroblast cultures

	Cell Density (OD 630 nm × 10³)			
Antibody	No TNF	0.1 ng/mL TNF	0.5 ng/mL TNF	2.0 ng/mL TNF
None	36 ± 1	50 ± 1	55 ± 2	63 ± 1
Control	35 ± 2	49 ± 1	54 ± 1	63 ± 1
cA2	39 ± 1	37 ± 1	39 ± 1	41 ± 2

FS-4 human fibroblasts were seeded in 96-well plates. Recombinant human TNF, preincubated with or without 4 μg/mL cA2 or control antibody, was added to the cultures and cell density was determined by staining 5 days later. Data represent the mean (± standard deviation) of quintuplicate wells.

tissue factor upregulation.[28] After the cells are lysed by freezing and thawing, PCA can be detected by measuring the clotting time of plasma to which calcium and cell lysate have been added. Figure 1 shows that while a clotting time of about 130 sec is obtained using an unstimulated HUVE lysate, addition of lysate from HUVE cells exposed to 25 ng/mL TNF for 4 h at 37°C shortens the clotting time by 50%. This reduction in clotting time was blocked by the addition of cA2 in the HUVE cell medium during the time of TNF exposure. A final cA2 concentration of 0.37 µg/mL could completely neutralize TNF-induced PCA while isotype-matched control IgG at 10 µg/mL had no effect.

TNF also acts on HUVE cells by inducing the cell surface expression of the adhesion proteins E-selectin[29] and ICAM-1.[30] Peak temporal expression of these surface antigens varies, however they can be individually quantified using specific monoclonal antibodies. The observed levels of TNF-induced E-selectin and ICAM-1 expression on the HUVE cell surface were reduced in a dose-dependent manner by the inclusion of cA2, while control IgG had no effect (Fig. 2). Induction of peak E-selectin expression (measured at 4 h) was fully abrogated by 0.5 µg/mL of cA2 (Fig. 2A), and complete blocking of peak ICAM-1 expression (at 23 h) required 0.1 µg/mL of cA2 (Fig. 2B).

In vivo, the expression of E-selectin and ICAM-1 by endothelial cells exposed to TNF results in the binding of circulating neutrophils and eventual extravasation into the surrounding tissues.[31] In order to determine if cA2 could effectively block the adhesion of neutrophils in an in vitro system, HUVE cell monolayers were first exposed to TNF in the presence and absence of cA2 for 4 h, then exposed to isolated human neutrophils. After washing, the percentage of neutrophils that adhered to the HUVE cells was determined using a neutrophil-specific myeloperoxidase assay. When treated with TNF alone, neutrophil adherence increased 52%, a fourfold increase over unstimulated HUVE cells (Fig. 3). Increasing amounts of cA2 reduced the degree of neutrophil adherence in a dose-dependent manner, while 3.3 µg/mL of control IgG had no effect. cA2 had no effect on neutrophil adherence to HUVE cells treated with IL-1 or LPS, demonstrating that the inhibitory effect of cA2 is directed specifically at TNF.

The inhibition of TNF-induced neutrophil adherence was also apparent by phase contrast light microscopy (Fig. 4). Neutrophils can be identified by their rounded, highly refractile appearance as opposed to the flat morphology exhibited by the HUVE cell monolayer. In Panel A, unstimulated HUVE cells show few adherent neutrophils compared to HUVE cells stimulated with TNF (Panel B). The presence of control IgG during TNF stimulation (Panel C) had no effect, while the presence of 3.3 µg/mL of cA2 during

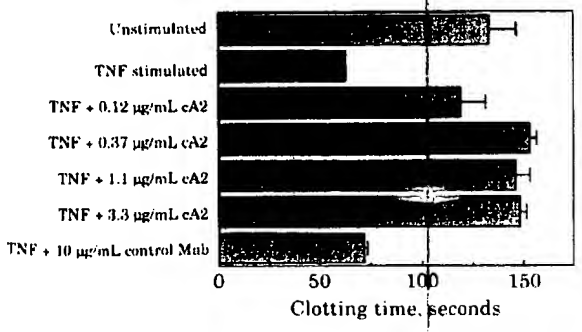

Figure 1. Effect of cA2 on the production of rhTNF-induced procoagulant activity by HUVE cells.

cA2 or control antibody were preincubated 30 min at room temperature with 25 ng/mL rhTNF prior to cell stimulation for 4 h at 37°C. Procoagulant activity was measured by determining the clotting time of recalcified human plasma after addition of the treated HUVE cell lysates. Data shown are the mean of duplicate wells ± SEM.

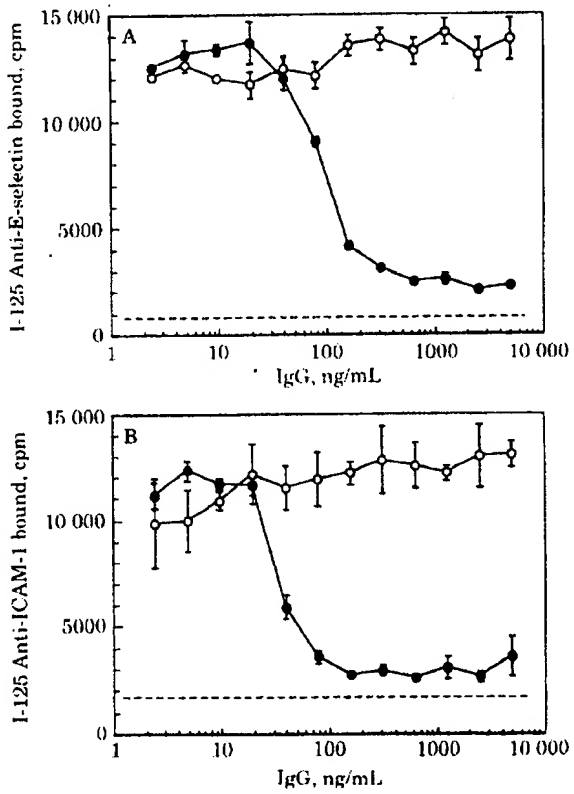

Figure 2. Neutralization of rhTNF-induced HUVE cell E-selectin and ICAM-1 expression by cA2.

Serial dilutions of cA2 (●) or control antibody (○) were mixed with 10 ng/mL of rhTNF and used to stimulate endothelial cell monolayers. E-selectin (Panel A) and ICAM-1 (Panel B) were detected after stimulation for 4 h and 23 h, respectively, using ^{125}I monoclonal antibodies specific for each adhesion protein. Data shown are the mean of triplicate wells ± SEM. The values obtained on unstimulated cells were 736 ± 65 cpm for E-selectin at 4 h and 1723 ± 149 cpm for ICAM-1 at 23 h (shown by dashed line).

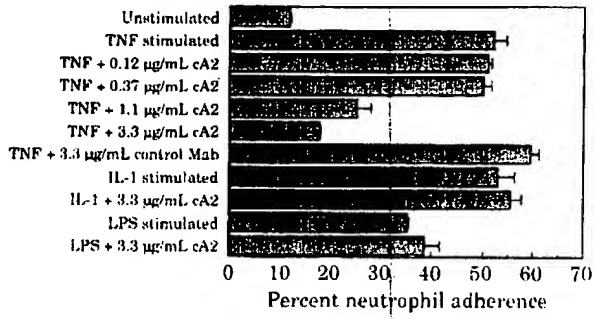

Figure 3. Neutralization of rhTNF-induced human neutrophil adhesion to HUVE cells by cA2.

HUVE cell monolayers (about 3×10^5 cells per well) were stimulated for 4 h with rhTNF (250 ng/mL), IL-1α (40 units/mL), J5 LPS (10 ng/mL) or medium alone (unstimulated). Incubations were performed in the presence or absence of the indicated concentrations of cA2 or control antibody. After washing, the stimulated HUVE cells were incubated an additional 45 min with human neutrophils (1×10^6 per well), and the number of neutrophils bound to the HUVE cells was determined by the myeloperoxidase assay. The data shown represents the average of triplicate wells ± SEM.

TNF stimulation (Panel D) markedly reduced the number of adherent neutrophils.

Effect of cA2 on TNF priming of human neutrophils

In vitro, TNF primes human neutrophils to produce superoxide upon subsequent stimulation with the chemotactic peptide f-met-leu-phe (FMLP).[32] The ability of cA2 to abrogate the TNF-priming phenomenon is shown in Figure 5. In the absence of cA2, TNF-primed (2 ng/mL) neutrophils produced as much as 40–45 nM superoxide upon stimulation with FMLP. TNF alone induced little or no superoxide production. cA2 was able to reduce the TNF-induced priming phenomenon in a dose-dependent manner, with levels as low as 1 μg/ml able to reduce superoxide production to the level typically seen with unprimed FMLP-stimulated neutrophils (7.85 nM in the experiment shown). Incubation of up to 100 μg/mL of control antibody with TNF had no effect on the subsequent production of superoxide upon FMLP stimulation (data not shown).

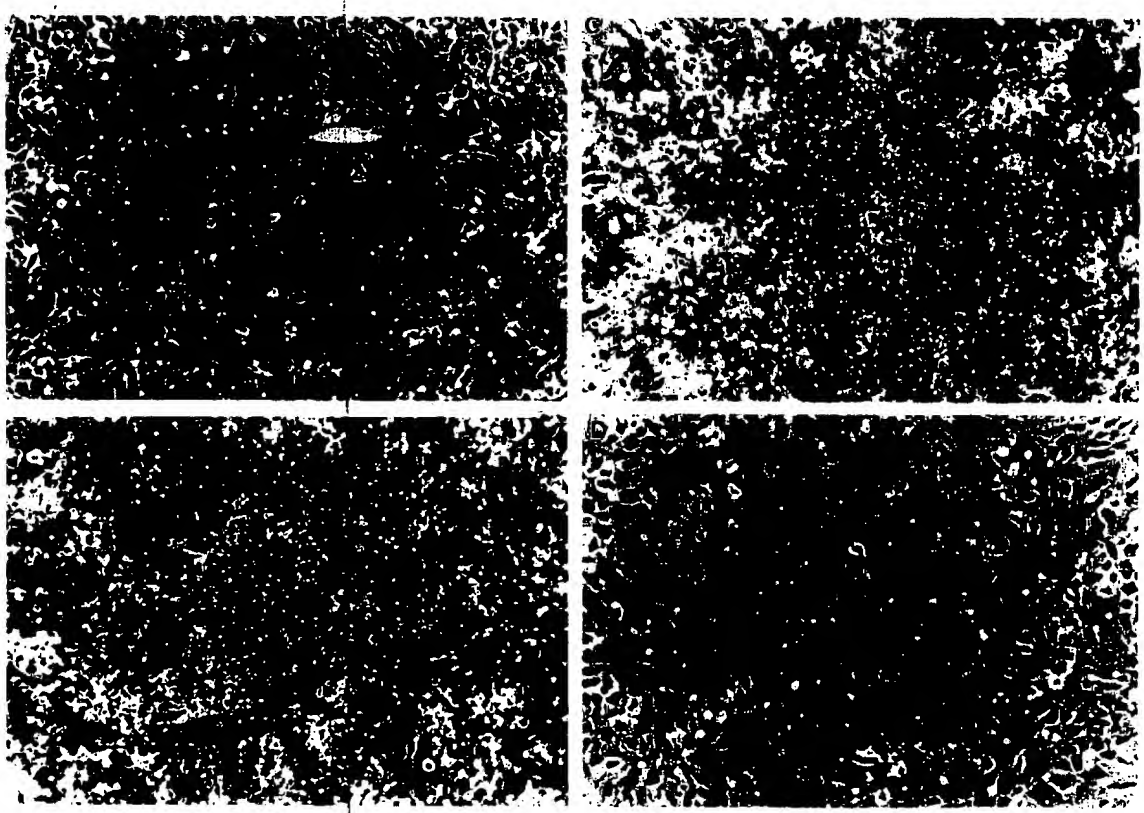

Figure 4. Phase contrast micrographs demonstrating neutrophil adherence to HUVE cells stimulated with rhTNF in the presence and absence of cA2.

HUVE cells were stimulated for 4 h with medium alone (A), 250 ng/mL rhTNF (B), 250 ng/mL rhTNF and 3.3 μg/mL control antibody (C) or 250 ng/mL rhTNF and 10 μg/mL cA2 (D). After stimulation, the ability of human neutrophils to adhere to the HUVE cell monolayer was assessed. Phase contrast micrographs were taken at 100-fold magnification.

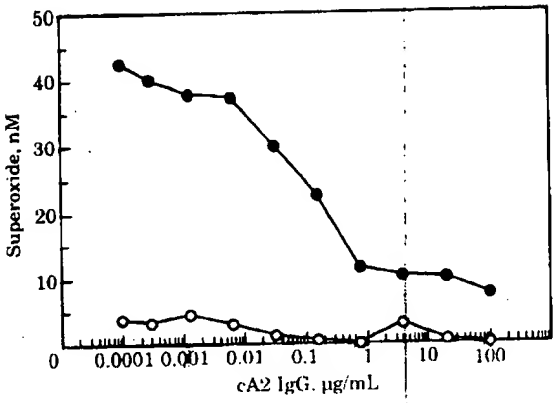

Figure 5. Effect of cA2 on the ability of rhTNF to prime human neutrophils.

Neutrophils (2.5×10^6 cells per mL) were primed with 2 ng/mL rhTNF in the presence of cA2 for 60 min, then the neutrophil activator FMLP (0.1 μM) (●) or medium (○) was added and the incubation continued for 10 min. Superoxide ion production was detected via the oxidation of cytochrome C. The data represent the average of duplicate wells with the background signal observed in parallel samples containing superoxide dismutase subtracted. Superoxide ion production by unprimed neutrophils exposed to FMLP was 7.85 nM.

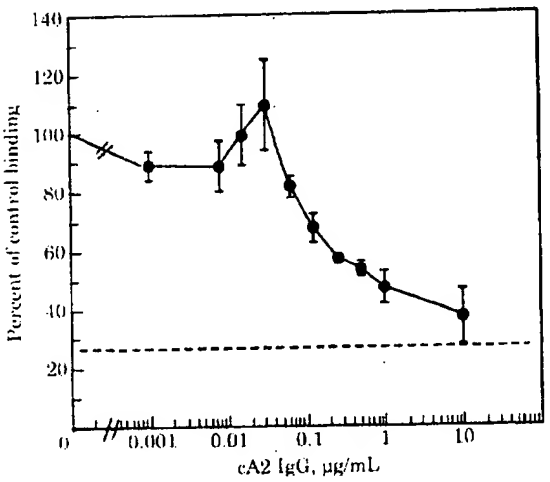

Figure 6. Binding of ^{125}I-rhTNF to U937 cell membranes in the presence of cA2.

U937 membranes were incubated for 3 h at 0°C with ^{125}I-rhTNF (45 pM) and the indicated concentration of cA2. ^{125}I-rhTNF bound to membranes was then separated by filtration and counted. Data are expressed as the mean ± SEM (three experiments) percent of binding in the absence of cA2 (1100 cpm). Binding of ^{125}I-rhTNF in the presence of 10 μg/mL negative control antibody was 96 ± 1%. Binding of ^{125}I-rhTNF in the presence of 40 nM unlabelled TNF (nonspecific background) was 26 ± 9% (shown by dashed line).

Effect of cA2 on TNF binding to receptor

The inhibition of TNF biological activity by cA2 presumably occurs as a result of the ability of cA2 to bind to soluble TNF, thereby inhibiting its interaction with cellular receptors. In order to directly demonstrate this in vitro, experiments were performed using both a commercially-available TNF receptor binding assay and recombinant immunoadhesion constructs of the human p55 and p75 TNF receptors. Figure 6 shows that cA2 inhibits the binding of radiolabelled TNF to a preparation of U937 monocytic cell membranes which contain TNF receptors. U937 cells have been previously shown to express both the p55 and p75 TNF receptors.[33] cA2 inhibition was dose-dependent, with 50% inhibition observed at 0.1 μg/mL.

Similarly, the binding of radiolabelled TNF to recombinant constructs containing the extracellular domain of either the p55 or p75 TNF receptor was inhibited by cA2 (Fig. 7). Inhibition by cA2 was dose-dependent, and 50% inhibition of binding to either receptor construct was evident at about 0.03 μg/mL cA2.

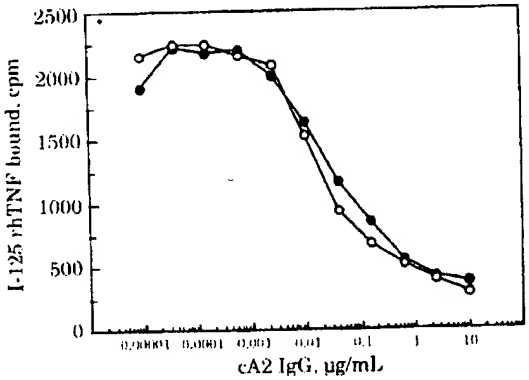

Figure 7. Binding of ^{125}I-rhTNF to p55 or p75 receptor fusion protein in the presence of cA2.

Serial dilutions of cA2 in 4 ng/mL ^{125}I-rhTNF were incubated on p55 (●) or p75 (○) receptor-coated microtiter wells for 1 h at 37°C. The data represent the mean of duplicate wells. Binding of ^{125}I-rhTNF in the presence of 10 μg/mL negative control antibody was 2270 cpm.

Effect of cA2 in a transgenic mouse model

Transgenic mice have been generated in which human TNF is constitutively expressed by their T cells.[25] These animals show elevated human TNF serum levels (0.01–7 units/mL) and develop a lethal wasting syndrome resulting in 80%–100% mortality at 10–18 weeks after birth. The ability of cA2 to prevent mortality in the transgenic mouse line Tg211 is shown in the survival curves of Figure 8. There was complete survival (15/15) to the 8 week endpoint in 3 week old animals administered cA2 twice-weekly at doses of 8 or 2 mg/kg. In the group administered the lowest dose of cA2, 0.5 mg/kg, there was a final 93% survival rate (14/15). By contrast,

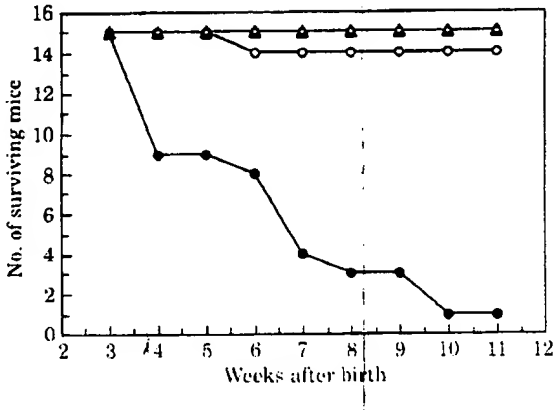

Figure 8. Survival curves of rhTNF transgenic mice treated with 0.5 (○), 2 (▲) and 8 (□) mg/kg cA2 IgG or 8 mg/kg control antibody (●).

Treatments were given twice weekly beginning at 3 weeks of age and continued for 8 weeks.

the control antibody group had a final survival rate of 7% (1/15). The difference between the least protective cA2 group (0.5 mg/kg) and the control group was highly statistically significant, log-rank $P = 0.0001$. The beneficial effect of cA2 was also evident when comparing weight gain between groups. Table 3 shows the average weight for each group at study entry (week 3), at study mid-point (week 7), and at study termination (week 11). Animals in the three cA2 treatment groups showed significant weight gain throughout the 8-week study which averaged 134%. By contrast, the control group showed only a modest (49%) weight gain throughout the 8-week study. Even this modest weight gain in the control group may be an overestimation since the control animals that died during the study may have experienced the least weight gain; however, they were obviously excluded from the later time points.

DISCUSSION

In this study we evaluated the effect of the chimeric anti-human TNF monoclonal antibody cA2 on both the in vitro and in vivo biological activities of TNF. cA2 exhibited potent neutralizing activity against TNF-mediated effects on human fibroblast, neutrophil and endothelial cell cultures. In the bioassays examined, 4 μg/mL of cA2 was sufficient to prevent the biological effects of an optimal challenge dose of TNF. The effects of cA2 were dose-dependent and highly specific. Incubation of cA2 with TNF directly inhibited the binding of TNF to receptor-containing cell membrane preparations and to recombinant constructs of the human p55 and p75 TNF cellular receptors. The neutralizing activity of cA2 could also be demonstrated in vivo, as evidenced by the ability of cA2 to reverse both the phenotype and accelerated mortality seen in transgenic mice that express the human TNF gene. In this case, cA2 efficacy was demonstrated in a chronic disease model by administering the drug over a period of 8 weeks. The potency of cA2 was evidenced by the suppression of mortality and weight loss with cA2 doses as low as 0.5 mg/kg administered twice weekly. These observations support and extend our previous studies which showed cA2 to be a high-affinity, highly-specific, TNF neutralizing antibody.[22]

The data presented for cA2 are consistent with the previously reported benefit of anti-TNF therapy in animal models of bacterial sepsis. Sepsis is a complex syndrome and it may be necessary to develop therapeutic modalities which target multiple disease mediators. Although clinical trials using passive anti-TNF immunotherapy are underway,[34] no efficacy data have been reported.

How do these findings relate to the potential application of cA2 to human autoimmune disease? In the case of RA, TNF has been implicated as a primary mediator of the chronic inflammation, although other

TABLE 3. Mortality and weight changes in TNF transgenic mice treated with cA2

Group	Study entry (week 3)	Study midpoint (week 7)		Study termination (week 11)	
	Average wt (g)	Number of Survivors	Average wt (g)	Number of Survivors	Average wt (g)
8 mg/kg cA2	7.4	15	16.0	15	20.0
2 mg/kg cA2	9.3	15	16.9	15	19.6
0.5 mg/kg cA2	7.6	14	15.9	14	16.8
8 mg/kg control antibody	10.0	4	11.4	1	14.9

Three week old Tg211 transgenic mice expressing rhTNF (n = 15 per group) were injected twice weekly with cA2 or an isotype-matched control antibody. Weights were measured weekly and represent the average of surviving animals.

inflammatory cytokines are also important.[16, 35] TNF has been shown in vitro to cause proliferation of synovial cells[36] as well as fibroblasts[26] which could directly contribute to pannus formation and fibrosis, respectively. This correlation is strengthened by the report that TNF is produced by cells at the cartilage-pannus junction[37] and is secreted spontaneously by cultured cells from synovial fluid from RA patients.[10] Thus, the ability of cA2 to block TNF-induced mitogenesis of human diploid fibroblasts, as well as their secretion of IL-6, may be relevant to this phenomenon. Pannus formation in diseased joints is accompanied by increased expression of ICAM-1,[14] which can be upregulated by several cytokines including TNF and IL-1, and leads to the infiltration of mononuclear cells. In the present study, cA2 has been shown to block TNF-induced ICAM-1 (as well as E-selectin) expression by cultured human endothelial cells. The blocking by cA2 of ICAM-1 and E-selectin expression directly resulted in decreased adherence of human neutrophils to endothelial cell monolayers. Neutrophils are numerous in synovial fluid from RA patients and it is likely that their activation leads to further necrosis of tissue. It may therefore also be important that cA2 can block the priming of neutrophils by TNF. Although not investigated in this study, TNF also is involved in the induction of collagenase in synovial cells,[38] the inhibition of prostaglandin synthesis by articular chondrocytes[39] and the stimulation of bone resorption.[40] Moreover, it has been shown that the use of an anti-human TNF monoclonal antibody completely neutralizes development of disease in a human TNF transgenic model of arthritis.[20] The potential for reducing RA joint disease using an anti-TNF antibody approach has also been demonstrated in a murine model of collagen-induced arthritis.[41] Mice were treated weekly with 10 mg/kg of anti-murine TNF antibody, with treatment beginning either before immunization with type II collagen or after immunization and onset of clinical arthritis. In both circumstances, the anti-TNF antibody reduced swelling of the paws and severity of disease by histopathological assessment of the arthritic joints.

While numerous cytokines such as TNF, IL-1, IL-6, GM-CSF and TGF-β have been identified in joint synovial fluids of patients with RA,[15] recent studies by Feldmann and colleagues suggest that TNF may regulate the levels of these other cytokines.[42, 43] Earlier studies have demonstrated that TNF is a potent inducer of IL-1 in endothelial cells[44] and monocytes,[45] as well as of IL-6[46] and GM-CSF.[47] Cells cultured from the synovial fluid from the diseased joints of RA patients continue to spontaneously produce bioactive IL-1. The addition of a polyclonal, neutralizing anti-human TNF antibody specifically reduced the levels of bioactive IL-1β produced.[44] In a similar set of experiments, levels of bioactive GM-CSF were shown to be significantly reduced by the addition of neutralizing anti-human TNF antibodies to the media of cultured synoviocytes from RA patients.[45] These results provide a clear rationale for the evaluation of anti-TNF therapy in RA, and results from initial studies to assess safety and efficacy in RA patients are encouraging.[24]

TNF also has been cited as a potential mediator in several other autoimmune diseases. Elevated serum levels of TNF correlate with relapsing ulcerative colitis and chronic Crohn's disease[48] and TNF in the stool of patients with inflammatory bowel disease may be a marker which correlates with disease activity.[49] A temporary remission has been described in a Crohn's patient treated with cA2.[23] In this case report, a Crohn's patient who was unresponsive to conventional treatment received two doses of cA2 (10 mg/kg) spaced two weeks apart. Over a period of ten weeks after treatment, the patient gained weight and showed reduction in standard indexes of disease activity as well as complete endoscopic remission. Symptoms returned approximately 3 months after treatment. Anti-TNF antibodies have also been evaluated in experimental allergic encephalomyelitis (EAE), an autoimmune demyelinating disease in mice which mimics multiple sclerosis. Treatment of mice with neutralizing anti-TNF antibodies prevented transfer of EAE symptoms by a T cell clone[50] and delayed relapse caused by bacterial superantigen.[51]

It should be pointed out that the precise molecular role(s) of TNF in each of these disease states remains to be elucidated, and that further understanding of localized versus systemic effects of this cytokine is critical to the rational design of targeted anti-TNF therapy. This is particularly important as the role of TNF may be beneficial in certain disease states and under certain conditions.[52] Highly specific, potent neutralizing agents such as cA2 provide a valuable tool for the elucidation of TNF biology in human disease, and may serve as new forms of therapeutic intervention in those cases where a causal relationship between aberrant TNF expression and disease pathology can be established. The results of the present study demonstrate the potential usefulness of cA2 and provide a rationale for its continued evaluation in human disease.

MATERIALS AND METHODS

Reagents

The monoclonal chimeric mouse/human anti-TNF IgG_1 (cA2) antibody was isolated from concentrated hybridoma cell supernatant by Protein A-Sepharose chromatography and ion exchange chromatography. Chimeric mouse/human 7E3 anti-platelet IgG_1, chimeric mouse/human 17-1A anti-tumour antigen IgG_1, and chimeric mouse/human MT-412 anti-CD4 IgG_1 were also purified by Protein A-Sepharose

chromatography and used as isotype-matched, irrelevant antibody controls. Lyophilized, carrier free recombinant human TNF (rhTNF) was obtained from Dainippon, Osaka, Japan; from Suntory, Osaka, Japan; and from Biosource, Camarillo, CA. The anti-E-selectin antibody H18/7 was a gift from Dr M. Bevilacqua, formerly at Brigham and Women's Hospital, Boston, MA. Anti-ICAM-1 antibody #11 was a gift from Dr G. Riethmuller, University of Munich, Germany. H18/7 and #11 antibodies were iodinated to a specific activity of 2–3 μCi/μg using Iodobeads (Pierce, Rockford, IL) as per the manufacturer's instructions. Human umbilical vein endothelial (HUVE) cells were obtained from Cell Systems, Kirkland, WA. The FS-4 fibroblast line was established and has been maintained at the New York University Medical Center. Recombinant constructs of the p55 and p75 human TNF receptors were a gift from Dr Bernard Scallon, Department of Molecular Biology, Centocor, Inc. Briefly, the p55-sf2 construct contains the extracellular domain of human p55 fused to eight amino acids of human antibody J sequence followed by all three constants domains of human IgG1. It is disulfide bonded to a human kappa light chain constant region. The p75P-sf2 construct is the same as p55-sf2 except it contains a truncated form of the extracellular domain of human p75. Both constructs were purified from cell culture supernatant by Protein A affinity chromatography.

Cell culture

Human umbilical vein endothelial (HUVE) cells were grown in complete HUVE medium (Cell Systems, Kirkland, WA) containing 15% fetal bovine serum (FBS; Hyclone, Logan UT) supplemented with growth factor (CS-HBGF-I/H; Cell Systems, Kirkland, WA) on T-75 tissue culture flasks (Corning, Corning, NY). All plasticware used for HUVE cell culture was coated first with attachment factor (Cell Systems, Kirkland, WA) by wetting the surface with attachment factor and removing the excess fluid prior to introducing cells. Cells were serially passaged by splitting 1:3 in the same medium every 3–5 days and all assays were performed on cells at passages 2–4.

FS-4 fibroblasts were grown in modified Eagle's medium (MEM) containing 5% FBS in T-75 flasks. Cells were serially passaged by splitting 1:5 in the same medium every 2 weeks; cells at passage level 13 to 15 were used in the experiments.

Mitogenesis assay

FS-4 fibroblasts were seeded at 8×10^3 cells/well in a 96-well tissue culture plate in MEM containing 5% FBS and cultured for 18 h at 37°C. Recombinant human TNF was diluted in complete MEM to final concentrations of 0, 0.1, 0.5 and 2 ng/mL in the absence or presence of 4 μg/mL test antibody. The samples were preincubated for 20 min at room temperature, then 0.1 mL was added to quintuplicate wells and incubated at 37°C for 5 days. The cells were rinsed with phosphate buffered saline (PBS) pH 7.2, then fixed by adding 50 μL/well of 10% formalin in PBS for 15 min at room temperature. The fixed cells were then stained with 50 μL/well of 0.05% naphthol blue black in 9% acetic acid, 0.1 M sodium acetate for 30 min at room temperature. The cells were then rinsed thoroughly with distilled water, and the bound dye was eluted with 150 μL/well of 50 mM NaOH. Absorbance of the eluted dye was determined at 630 nm.

Assay for IL-6

FS-4 fibroblasts were seeded at 2×10^4 cells/well in a 96-well tissue culture plate in MEM containing 5% FBS and cultured overnight at 37°C. Recombinant human TNF was diluted to final concentrations of 0, 0.3, 1.5 and 7.5 ng/mL in the absence or presence of 4 μg/mL test antibody. The samples were preincubated for 20 min at room temperature, then 0.1 mL was added to duplicate culture wells and the incubation continued for 18 h. The cell supernatants from duplicate wells were pooled and stored at -20°C. The amount of IL-6 present in each sample was determined using an ELISA-based plate assay (Quantikine IL-6, R&D Systems, Minneapolis, MN) as described by the manufacturer.

Procoagulant activity assay (PCA)

The PCA assay was performed on HUVE cells plated at 1.5×10^5 cells/well in 24-well tissue culture plates (Falcon) coated with attachment factor. Confluent monolayers were washed three times with 500 μL/well of RPMI 1640 (JRH Biosciences, Lenexa, KS) containing 1% FBS and 0.3 mg/mL L-glutamine (JRH Biosciences, Lenexa, KS). Antibody samples were tested at 3.3, 1.1, 0.37 and 0.12 μg/mL for neutralization of rhTNF at a final concentration of 25 ng/mL. Medium alone (no rhTNF) and medium plus 10 μg/mL cA2, were used as negative controls while medium containing rhTNF at 25 ng/mL (no antibody) was used as a positive control. All dilutions were prepared in complete RPMI medium and were preincubated for 30 min at room temperature prior to incubation with the endothelial cells. Test solutions were dispensed into duplicate wells, 500 μL/well, and incubated for 4 h at 37°C. The test solutions were removed with gentle aspiration and the cells were washed as described previously. Three hundred microliters of complete RPMI were dispensed into each well and the plates were immediately frozen at -70°C. Cell lysates were prepared by thawing the plates at room temperature, resuspending all cell debris, and freezing and thawing each plate two more times. The plasma clotting assay was performed after equilibrating all reagents at 37°C. Clotting was initiated by mixing 0.1 mL of fresh, citrated human plasma, 0.1 mL of cell lysate and 0.1 mL of 30 mM $CaCl_2$ in a glass tube and incubating at 37°C. The time required for the clot to form (by visual inspection) was recorded and the mean and standard deviation from duplicate cell lysates were calculated.

Adhesion protein assays

The E-selectin and ICAM-1 assays were performed on HUVE cells plated at 5×10^4 cells/well in 96-well tissue culture plates (Costar 3596, Cambridge, MA) coated with attachment factor. Confluent monolayers were gently washed twice using a multi-channel pipettor with 150 μL/well of HUVE medium. Twofold serial dilutions of cA2 or c17-1A were prepared in medium containing 10 ng/mL rhTNF. Medium alone was used as a negative control while medium containing 10 ng/mL rhTNF was used as a positive control. Test solutions were dispensed into triplicate wells, 100 μL/

well, and incubated for 4 h (E-selectin assay) or 23 h (ICAM-1 assay) at 37°C. The test solutions were removed with a multichannel pipettor and the iodinated probes were added. Expression of adhesion protein was detected by incubating the cells for 2 h at room temperature with ^{125}I-anti-E-selectin antibody H18/7 F(ab')$_2$ or ^{125}I-anti-ICAM-1 #11 IgG diluted to 10 µg/mL in complete HUVE medium (300000 cpm/100 µL/well). The cells were then washed 4× with RPMI-1640 containing 10% FBS, and the well contents solubilized and counted for ^{125}I in a gamma counter.

Neutrophil adhesion and priming assays

Neutrophils were isolated from 100 mL of fresh human blood drawn into heparin. Ten millilitres of blood were layered onto 5 mL of Mono-poly resolving medium (Flow Labs, McLean, VA) in a 15 mL conical centrifuge tube and centrifuged at 750 × *g* for 30 min at room temperature. Additional centrifugation for 30 min at 900 × *g* was generally required to completely separate the bands of cells. The top band containing T and B cells was discarded and the lower band containing polymorphonuclear cells was collected. The neutrophils were washed with Hank's buffered saline (without magnesium and calcium) and were resuspended in RPMI-1640/10% FBS.

For the adhesion assay, HUVE cells grown in 24-well tissue culture plates were stimulated for 4 h at 37°C with rhTNF (250 ng/mL), IL-1α (40 units/mL; Genzyme, Boston, MA) or *E. coli* J5 LPS (10 ng/mL; List Biological Laboratories, Campbell, CA) in RPMI-1640/10% FBS containing the indicated concentration of test antibody, or with medium alone. The HUVE monolayers, containing about 3×10^5 cells per well, were then washed once and overlaid with 0.1 mL of RPMI-1640/10% FBS containing 1×10^6 neutrophils. After incubation for 45 min at 37°C, the monolayers were gently washed three times with RPMI-1640/10% FBS and then solubilized with 0.25 mL of 50 mM potassium phosphate pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The solubilized monolayer samples were then quantitated using the myeloperoxidase assay in which known quantities of isolated neutrophils were processed in the same manner and used to generate a standard curve. Standard samples were analysed in duplicate while test samples were tested in triplicate. Reagents were added to the wells of a 96-well microtiter plate in the following order: 55 µL of 80 mM potassium phosphate pH 5.4; 15 µL of sample, standard or blank; 20 µL of 0.3 mM hydrogen peroxide in 80 mM potassium phosphate pH 5.4; 10 µL of 16 mM 3,3',5,5'-tetramethylbenzidine in N, N'-dimethylformamide. The plate was incubated for 15 min at room temperature and the reaction stopped by adding 100 µL of 1 M phosphoric acid per well. The optical density of each well was read at 450 nm. Photomicroscopy of HUVE cell monolayers at 100× magnification was performed using a phase contrast Nikon IMT-2 inverted research microscope.

For neutrophil oxidative burst assays, 2.5×10^6 cells/mL were primed with 2.0 ng/mL rhTNF in the presence of the indicated concentration of test antibody for 60 min at 37°C. Primed cells were activated (or mock-activated) with 0.1 µM FMLP (Sigma, St. Louis, MO) for 10 min at 37°C in the presence of 1 mg/mL cytochrome C (Sigma, St. Louis, MO). The cells were then microcentrifuged for 5 min and the OD of the supernatants read at 550 nm. Duplicate samples containing 10 µg/mL superoxide dismutase (SOD; Sigma) were run in parallel and the background OD obtained subtracted from samples without SOD. The results were converted to nM superoxide ion using the extinction coefficient for (reduced cytochrome c) - (oxidized cytochrome c) for a 3 mm path length.[53]

Receptor binding assays

^{125}I-rhTNF binding to U937 membranes was performed using a New England Nuclear (Boston, MA) ligand binding kit. Briefly, 45 pM of ^{125}I-rhTNF (40–50 µCi/µg), the indicated concentrations of test antibody and U937 membranes provided, were incubated as described in the manufacturer's instructions in a final volume of 250 µl for 3 h on ice. Membrane bound tracer was separated from free tracer by vacuum filtration over GF/C filters. The filters were washed 2 × 4 ml with the wash buffer provided. Data were expressed as the mean ± SEM of three separate experiments and graphed as percent of control ^{125}I-rhTNF binding in the absence of antibody (approximately 1100 cpm). Binding of tracer in the presence of 10 µg/ml of a negative control antibody was 96% ± 1%.

To assess ^{125}I-rhTNF binding to recombinant constructs of the p55 and p75 cellular receptors for TNF, 50 µL of a 5 µg/mL solution of either p55 or p75 receptor fusion proteins in PBS was incubated on polystyrene 96-well plates for 1 h at 37°C. The wells were washed and blocked for 1 h at 37°C with PBS containing 1% BSA. Equal volumes (25 µL each) of serially diluted cA2 in PBS/1% BSA and 2 × ^{125}I-rhTNF (final concentration = 4 ng/mL) were added to duplicate wells. Plates were incubated for 1 h at 37°C, washed 2 × 200 µL with PBS and the radioactivity bound was counted in a gamma counter. Binding of tracer in the presence of 10 µg/mL negative control antibody was 2270 cpm.

Transgenic mouse protection model

Tg211 transgenic mice were bred as previously described[19] and randomly divided into groups of 15 animals each. These groups received twice-weekly intraperitoneal injections of 10 µL per gram average body weight to achieve a final dose of 0.5, 2 and 8 mg/kg cA2 IgG. A fourth group received 8 mg/kg of an isotype-matched control antibody. Investigators (LP and GK) performing the experiment at the Hellenic Pasteur Institute were blinded with respect to the drug each treatment group received during the course of the study. Injections of test antibody were initiated when the mice reached 3 weeks of age, and the study was terminated after 8 weeks of treatment. Average weight and mortality in each group was recorded weekly.

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